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Rare independent mutations in renal salt handling genes contribute to blood pressure variation

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Abstract

The effects of alleles in many genes are believed to contribute to common complex diseases such as hypertension. Whether risk alleles comprise a small number of common variants or many rare independent mutations at trait loci is largely unknown. We screened members of the Framingham Heart Study (FHS) for variation in three genes -SLC12A3 (NCCT), SLC12A1 (NKCC2) and KCNJ1 (ROMK)- causing rare recessive diseases featuring large reductions in blood pressure. Using comparative genomics, genetics, and biochemistry, we identified subjects with mutations proven or inferred to be functional. These mutations, all heterozygous and rare, produce clinically significant blood pressure reduction and protect from development of hypertension. Our findings implicate many rare alleles that alter renal salt handling in blood pressure variation in the general population, and identify alleles with health benefit that are nonetheless under purifying selection. These findings have implications for the genetic architecture of hypertension and other common complex traits.

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GenBank mRNA sequences are as follows: *NCCT/SLC12A3* (NM_000339); *NKCC2/SLC12A1* (NM_000338); *ROMK/KCNJ1* (NM_000220, NM_153764-7). GenBank protein sequences are as follows: SLC12A3 orthologs: Human (NP_000330), mouse (NP_062288), rat (NP_062218), rabbit (AAC33139), dog (XP_535292), cow (XP_871112), chicken (XP_414059), zebrafish (NP_001038545) and winter flounder (AAL26926); SLC12A1orthologs: Human (NP_000329), mouse (NP_899197), rat (NP_062007), rabbit (AAB03494), dog (XP_850426), chicken (XP_413814), zebrafish (NP_001002080) and *Tetraodon* (CAF99849); SLC12A1-3 invertebrate orthologs: *S. purpuratus* (XP_783014), *C. elegans* (NP_502704) and *D. melanogaster* (NP_648572); KCNJ1 orthologs: Human (NP_72245), mouse (NP_062633), rat (NP_058719), dog (XP_546403), chicken (XP_425795), cow (XP_585917), frog (AAH79788), zebrafish (XP_684541), fugu (ABB87033), *C.elegans* (NP_509138), *S. purpuratus* (XP_789112) and *D. melanogaster* (NP_651131); Other human paralogs: SLC12A2 (NP_001037), SLC12A4 (NP_005063), SLC12A5 (NP_065759), SLC12A6 (NP_005126) and SLC12A7 (NP_006589), KCNJ2 (NP_000882), KCNJ3 (NP_002230) KCNJ4 (NP_004972), KCNJ5 (NP_000881), KCNJ6 (NP_002231), KCNJ8 (NP_004973), KCNJ9 (NP_004974), KCNJ10 (NP_002232), KCNJ11 (NP_000516), KCNJ12 (NP_066292), KCNJ13 (NP_002233), KCNJ14 (NP_733838), KCNJ15 (NP_733933) and KCNJ16 (NP_733938).

Hypertension affects 1 billion people world-wide and is a major contributor to death from stroke, myocardial infarction, end-stage renal disease and congestive heart failure. Although epidemiologic studies have demonstrated high heritability of blood pressure variation (h^2 of long-term blood pressure ~0.6)[1], identification of the responsible genes has been difficult owing to the trait's complexity.

Whether common variants or, alternatively, many independent rare mutations will accounted for the contributions of specific genes is unknown. Recent large genome-wide association studies have identified a number of common variants that contribute to metabolic traits including diabetes [2-4], coronary artery disease [5,6], and obesity[7]; notably, these collectively explain only a small fraction of inter-individual risk[2]. These studies have thus far failed to identify significant loci for blood pressure or hypertension[2,8], raising the possibility that rare independent variants account for a large fraction of blood pressure variation, as would be expected if trait alleles are under purifying selection[9].

The study of rare Mendelian traits has identified more than 20 genes in which mutations impart large effects on blood pressure; these predominantly act by changing net renal salt reabsorption[10,11]. Several of these disorders, exemplified by Bartter's and Gitelman's syndromes, are recessive traits that lower blood pressure, raising the question of whether the more prevalent heterozygous mutations in these genes might commonly affect the trait. Bartter's syndrome features massive renal salt wasting and hypotension, often resulting in neonatal death. It is caused by recessive loss of function mutations in any of 4 genes required for normal renal NaCl reabsorption[12-15]. These include the Na-K-2Cl cotransporter *SLC12A1* and the inward rectifier K^+ channel *KCNJ1.* Gitelman's syndrome is a less severe salt wasting disease caused by recessive loss of function mutations in the Na-Cl cotransporter *SLC12A3* [16].

The prevalence of Bartter's and Gitelman's syndromes (estimated at ~1 per million and 1 per 40,000, respectively)[16,17] suggest that heterozygous disease alleles should be present in at least 1% of the population (likely an underestimate owing to early lethality of Bartter's syndrome), motivating evaluation of these genes in a large well-characterized cohort. We examined *SLC12A1, SLC12A3* and *KCNJ1* in the Framingham Heart Study (FHS) offspring cohort. This cohort, comprising 5,124 subjects (3,125 with DNA), has been followed for up to 35 years with periodic evaluation of cardiovascular risk factors and other traits, permitting stable assessments of quantitative trait values over time.

RESULTS

Mutations in *SLC12A1, SLC12A3* and *KCNJ1* in FHS

We screened all coding exons and flanking intronic sequences of *SLC12A1, SLC12A3* and *KCNJ1* for DNA sequence variants in the 1,985 unrelated subjects and 1,140 relatives with available DNA samples. This constituted evaluation of ~24 Mb of diploid sequence. The sensitivity of variant detection was high, as we detected all 18 known SNPs in these genes and their allele frequencies in FHS were similar to previous estimates (Supplementary Table 1 online). In addition, eight subjects with Gitelman's syndrome were blindly included in the screen and their 16 known mutations were correctly identified. Variants were confirmed by two independent methods (Supplementary Fig. 1 online) and variants in the final set were further verified by independent amplification of the original sample. These variants recurred among siblings at expected Mendelian frequencies (32 of 60 siblings), providing assurance of proper sample tracking and indicating that few variants are *de novo* mutations.

A total of 138 different coding sequence variants were found in 2,492 FHS subjects, wheres the remainder showed only wild-type sequence (Supplementary Table 2 online). These

included 46 different synonymous substitutions found in 2235 subjects, 89 different missense variants found in 1062 subjects, one nonsense, and two frameshift mutations.

Given the rarity of Bartter and Gitelman syndromes, the vast majority of these variants must not cause loss of function. We first identified well-documented disease causing mutations which had been shown biochemically to cause loss of function of the encoded cotransporter/channel[18-22]. This identified 10 different biochemically proven loss of function mutations found in 23 subjects (Table 1).

We next sought to identify additional functional variants using phylogenetic conservation as indication of amino acid positions that are subject to purifying selection. We characterized orthologs and paralogs of each gene from diverse vertebrate and invertebrate species (10-12 orthologs and 6-14 paralogs for each gene, see Methods) and determined the conservation of each amino acid in each encoded protein. The proportion of amino acids that are completely conserved among orthologs ranges from 18% to 24% for each gene; non-conserved positions have an average of 3.5 ± 1.1 different variants, demonstrating substantial branch length in this set.

Similarly, variants that are under strong purifying selection are expected to be found at low frequency in the population; we used allele frequency < 0.001 as a threshold (using a threshold of < 0.01 would have yielded the identical set of variants). This criterion is supported by empiric data in our population, as the previously identified functional mutations that are found in FHS all have allele frequencies ≤ 0.0005.

As a critical test of the utility of these criteria, we determined their ability to identify disease-causing mutations in 148 unrelated subjects with Gitelman syndrome (GS) and 144 with Bartter syndrome (Supplementary Table 3 online). These criteria identified 62 different disruptive mutations or missense mutations at highly conserved positions that accounted for 189 mutant alleles in subjects with Gitelman syndrome. From these data we calculate that these criteria provide 77% sensitivity and 90% specificity for identification of functional mutations. Further, the fact that 35 of the independent mutations in the Gitelman cohort were only found once indicates that saturation for disease-causing mutations has not been approached. Similar results were found for NKCC2 and ROMK (disruptive mutations or missense mutations at highly conserved positions are found in 86% and 65% of Bartter alleles respectively). It is worth noting that similar results are obtained if analysis is confined to the 128 Gitelman syndrome subjects who are Caucasian of European ancestry (criteria show 78% sensitivity and 90% specificity), excluding a strong effect of genetic background on the spectrum of functional mutations. These findings provide direct experimental validation that these criteria are sensitive and specific for identification of functional mutations in these genes (Supplementary Fig. 2 online; Supplementary Table 3 online; see Methods).

Applying these validated criteria- complete conservation and rare allele frequency- to identify mutations under purifying selection in FHS, we identified 19 additional missense variants found in 25 subjects; all are at completely conserved positions and have allele frequency < 0.0005. In addition, we included one further variant in *NCCT* found once in FHS, R861C, which has previously been reported as disease-causing[23,24] and was found in 4 of our European Caucasian GS subjects (homozygous in 1). This non-conservative substitution is at a position conserved among all vertebrates.

Reduced blood pressure among mutation carriers

The final set of functional variants includes 30 different mutations in 49 subjects: their characteristics are shown in Table 1 and Figure 1. Virtually all of these are non-conservative

substitutions and are predicted to be damaging by PolyPhen[25], SIFT[26], and PANTHER[27] but represent a small subset of the variants predicted as functional by these programs. All of these mutations have allele frequency ≤ 1 per 2000 in FHS.

The standardized age and sex-adjusted long-term average systolic and diastolic blood pressure in the entire FHS offspring cohort was determined as defined previously[1]. The long-term blood pressure values among mutation carriers are indicated in Figure 2. Eighty percent of mutation carriers had long-term SBP below the mean of the cohort (P = 0.001); similar results are obtained if one separately considers the biochemically defined (78% below mean) or inferred functional mutations (81% below mean; Table 2). There is an excess of mutation carriers among those in the lowest decile of SBP compared with the highest decile; this excess becomes increasingly significant as one compares the lowest to the highest 20%, 30%, 40% and 50% of the distribution (Table 3). This result is consistent with the expectation that the power to detect the impact of alleles of moderate effect is increased as one analyzes the complete distribution rather than simply the extremes[28].

Quantitatively, the mean long-term SBP among mutation carriers was 6.3 mmHg lower than the mean of the cohort (P = 0.0009); similarly reduced blood pressures are seen in separate analysis of subjects with biochemically proven mutations (6.8 mmHg reduction) and the genetic/genomically identified mutations (5.9 mmHg reduction; Table 2). Similar results are found for DBP, with mean effect of -3.4 mmHg (P = 0.003). Effects were not significantly different among the 3 genes studied, though there was a trend toward larger *ROMK* effects. There were no significant differences in the magnitude of effects in males and females, and there was no significant difference in body mass index between carriers and non-carriers. Altered blood pressure was not found among carriers of other classes of rare or common synonymous or non-synonymous variants (Supplementary Table 4 online).

Mutation carriers showed reduced blood pressure from the earliest age measured to the last. Mean SBP was 5.7 mmHg lower at age 40 (P = 0.02), 6.4 mmHg at 50 (P = 0.01) and 9.0 mmHg at 60 (P = 0.0002) (Fig. 2c). Blood pressure effects were significant and of similar magnitude for biochemically proven and inferred mutations (e.g. -9.8 and -8.6 mmHg, respectively, at age 60; Table 2). Although less than half the cohort had values measured after age 60, the magnitude of the effects remained large (SBP 11.5 mmHg lower; P = 0.03). Similar effects are seen on diastolic blood pressure (Fig. 2d).

As an independent test of the effect of these mutations, we compared the blood pressures of siblings in FHS who were discordant for mutations. This within-family test of linkage is independent of the association tests. Under the null hypothesis, carrier and non-carrier siblings should have blood pressures that are not significantly different from one another. Among 43 sibling pairs discordant for mutations, mutation carriers had mean SBP 6.6 mmHg lower than their non-carrier sibs (P = 0.009), providing further evidence of the impact of the carrier state on blood pressure (sibships with biochemically proven mutations account for 29 of these discordant sib pairs, and these mutation carriers show SBP 9.2 mmHg lower than non-carriers, P =0.002). Similarly, the blood pressure variance among carrier/carrier pairs was lower than the variance among carrier/non-carrier pairs (P = 0.01). Finally, among the 15 sibling pairs concordant for mutations, in 12 both sibs had long-term blood pressures below the mean of the cohort (Fig. 3; P = 0.002) and their mean systolic blood pressure was significantly lower than the mean value of non-carrier pairs (11.5 mmHg reduction, P = 0.0001). Eleven of these pairs were biochemically proven mutations and these showed a mean reduction of 11.6 mmHg (P = 0.001).

Reduced prevalence of hypertension among mutation carriers

Mutation carriers were significantly protected from development of hypertension (systolic or diastolic BP greater than 140 or 90 mmHg, respectively, and/or treatment with antihypertensive medication for hypertension). Kaplan-Meier analysis of the likelihood of developing hypertension by age 60 showed a 59% reduction in the risk of hypertension among mutation carriers compared with non-carriers (Log-rank P<0.003; 95% confidence interval 23%-71%) (Fig. 4a). Evidence of protection was seen at each age (Fig. 4b), and males and females showed no significant differences in the reduction in risk conferred by the carrier state (males n = 25, 60% reduction in risk; females n = 24, 57% reduction in risk). Carriers of biochemically proven and inferred mutations showed similar reductions in hypertension risk (45% and 68%, respectively; Table 2).

DISCUSSION

A large prior body of work has demonstrated that homozygous loss of function mutations in genes whose products mediate or regulate renal salt reabsorption result in reduced blood pressure in humans. These observations have begged the question of whether the more prevalent heterozygous state for mutations in these same genes might have significant effects in the population. We have shown that the carrier state for rare functional mutations in *NCCT, NKCC2* and *ROMK* reduce blood pressure in FHS participants. Each of these genes governs renal salt handling, and homozygous loss of function mutations in them lower blood pressure by reducing salt reabsorption; heterozygous mutations in NCCT have also been shown to increase renal salt loss[29].

We infer that the heterozygous mutations we identified lower blood pressure via their effects on renal salt reabsorption. Importantly, these results establish the role of altered renal salt handling in blood pressure variation in a general population.

The functional significance of identified mutations is strongly supported by comparative genomics, Mendelian genetics, and biochemical evidence. Nearly half of the mutation carriers (23 of 49) have mutations that are proven by biochemical assay to be loss of function, while the remainder are inferred to be functional by rigorous genetic and genomic criteria. While it is possible that some of our identified mutations might not be loss of function, our empiric data indicate that this should be an uncommon event; moreover, inclusion of such neutral or gain-of-function alleles should bias toward the null, rather than toward a false-positive result. Finally, separate analysis of each of these two groups demonstrates significant effects on blood pressure in both, and the magnitude of these effects are not significantly different from one another (Table 2). The phenotypic effects of these mutations are significant by tests of association with long-term and age-specific blood pressure for both systolic and diastolic blood pressure, as well as by Kaplan-Meier assessment for risk of development of hypertension. In addition, the within-family test of linkage, contrasting blood pressures of siblings, provides independent evidence of the significant impact of these mutations on blood pressure.

At least one in 64 FHS members carries a functional mutation in one of these 3 genes. This is likely an underestimate given the stringent criteria applied and the expectation that ~15% of deleterious mutations lie outside the coding regions [30]. The effects of the carrier state are relatively large, similar in magnitude to those of clinically-used antihypertensive agents [31]; these mutations reduce the risk of hypertension at age 60 by nearly 60% and, based on epidemiologic observations in the Framingham cohort, are predicted to reduce the risk of stroke by 40% [32,33] and acute coronary syndrome by 15% [34,35]. These effects are thus relevant to individual risk prediction and underscore the importance of these findings for public health.

The estimated prevalence of *NCCT* mutations in unrelated members of FHS is 0.48%, convergent to the estimated population frequency of Gitelman syndrome of ~1 per 40,000. In contrast, the allele frequencies for *NKCC2* and *ROMK* mutations are estimated to be 1/360 and 1/670, respectively, which leads to an estimated frequency of Bartter syndrome due to these two genes of ~1/100,000, which is much higher than the observed prevalence of Bartter syndrome in the population[17]. The likely explanation is early lethality of the homozygous state for these mutations. Unlike Gitelman syndrome, Bartter syndrome is well-recognized to be an early lethal disease, with death both during fetal development and the neonatal period, and with few survivors beyond age 10 owing to myriad complications and development of renal failure[36-38].

Similar findings of multiple rare mutations in genes for Mendelian recessive forms of HDL level variation [39], and rare variants contributing to altered triglyceride levels[40] have been reported, suggesting that such findings will not be infrequent. It is axiomatic that for genes subject solely to purifying selection, rare independent mutations, and not common functional variants, will predominate; these considerations indicate practical limitations to genome-wide association studies. These findings suggest that the re-sequencing of genes validated by studies of rare Mendelian traits or other methods and eventually the re-sequencing of all genes or entire genomes will provide important contributions to understanding the inherited contributions to common traits. These findings do not, however, exclude potential effects of common variants on these or any other traits.

In contrast to prior study of rare variants in HDL, while homozygous mutations in each of the genes we studied likely impair reproductive fitness, the heterozygous state is expected to confer health benefit in post-reproductive ages. This expectation is supported both by epidemiologic studies of the relationship of blood pressure and mortality, as well as by randomized controlled trials which document reduced mortality among patients treated with thiazide diuretics, pharmacologic inhibitors of NCCT [41]. These findings thus define a set of alleles that are inferred to reduce morbidity and mortality late in life, but are nonetheless under strong purifying selection because of adverse effects of the homozygous state. As this cohort ages, it will be of future interest to determine whether cardiovascular morbidity and mortality is in fact reduced among carriers.

We have shown that the combination of high conservation and low allele frequency provides both high sensitivity and high specificity for functional mutations in these genes (Supplementary Fig 2 online). In contrast, prediction programs such as SIFT often overestimate functional alleles because they fail to take into account allele frequencies. In our data set, SIFT predicted 43 different functional variants in 340 cohort members, and the effects of these variants were not significant (P = 0.15 for SBP); the lack of specificity of these prediction programs for functional mutations is well-recognized[42]. The results of this study underscore the importance of recognizing functional variants within a large background of neutral variants. The combination of phylogenetic conservation and rare allele frequency we employed was effective, and likely conservative- these criteria will not capture variants that are present at higher allele frequencies owing to balancing selection or non-equilibrium states. These results suggest that similar criteria may be more broadly useful in identifying functional mutations from raw sequence data.

It is worth noting that in our study, sequencing the entire cohort substantially increased the significance of our result compared with what would have been seen had we merely sequenced the tails of the distribution (Table 3). This is expected if the effect size attributable to mutations is moderate, rather than large[28]; this point should be carefully considered in the design of future studies. Similarly, as expected for variants with moderate

effect size, the distribution of blood pressures remains quite variable among carriers, presumably reflecting influences of other genetic and environmental factors.

The three genes studied were selected in part because *NCCT* and *NKCC2* are targets of the most commonly used diuretics, the thiazides and furosemide, respectively. Despite the much greater salt wasting seen in individuals homozygous for mutations in *NKCC2*, heterozygous mutations in these two genes result in similar reductions in blood pressure. Dosage compensation at *NKCC2* has been seen in heterozygous null mice, providing a potential explanation [43]. The study of ROMK was motivated in part by its potential as a new antihypertensive diuretic agent that might not produce the hypokalemia seen with the above diuretics[11]; the carrier state for *ROMK* shows effects at least as large as those seen among carriers of NCCT and NKCC2 mutation, providing encouragement for further investigation.

Finally, these findings have implications for the genetic architecture of blood pressure variation. We estimate that ~100 million subjects world-wide harbor loss of function mutations in these genes, and that these subjects are significantly protected from hypertension and its consequences; the overall prevalence of hypertension is inferred to be reduced by about 1% owing to their effects. Because these 3 genes comprise only a small fraction of those in which mutations are known to alter blood pressure [10], and because there are likely many additional genes yet to be discovered, it seems probable that the combined effects of rare independent mutations will account for a substantial fraction of blood pressure variation in the population.

METHODS

Human subjects

Clinical data and DNA samples from 3125 subjects of the offspring cohort of the Framingham Heart Study, as well as 292 subjects with Gitelman and Bartter syndrome were used in this study (see Supplementary Note). The study was approved by the Yale Human Investigation Committee and the Framingham DNA Committee.

Identification of genetic variation

The coding exons and their flanking splice sites of *NCCT, ROMK* and *NKCC2* were amplified from DNA samples using specific primers to direct polymerase chain reaction (PCR; 58 amplicons in total). Multiplex PCR was carried out using HotStart Taq polymerase (Qiagen) using 30 ng DNA in 5 μl reactions (Supplementary Table 5 online). The PCR products were then diluted, heat denatured, then slowly cooled, permitting heteroduplex formation as recommended by the manufacturer. Sequence variants were identified by temperature gradient capillary electrophoresis (TGCE) using a Spectrumedix instrument[44]. The PCR failure rate averaged ~1% across all amplicons. The DNA sequence of identified variants was determined by direct DNA sequencing of both strands using an ABI377 instrument; mutations in the final functional variant set were confirmed by independent amplification and sequencing from the original DNA sample.

Longitudinal blood pressure traits

Long-term average systolic blood pressure (SBP) and diastolic blood pressure (DBP) was calculated including adjustment for the effects of age, sex and treatment for hypertension as previously described[1]. In brief, SBP readings taken at exams between 25-75 years of age, and DBP readings taken between 25-54 years of age (because DBP declines with age, beginning around age 55 [1,45]) were used in the calculation of the longitudinal readings. Treatment for hypertension was taken into account by calculating an adjusted residual[1]. These long-term average values were adjusted for mean age in a linear regression, done

separately for males and females. The standardized residuals were used as quantitative traits. Additive heritability estimates were calculated using the program SOLAR. Heritability estimates (h^2) for long-term average SBP was 0.66, and DBP was 0.60, which were each higher than that for any single examination estimate. For analyses at age 40, 50, 60 and 70, trait values at the last examination within each age range (25-40, 41-50, 51-60 and 61-70) were used. Numbers of individuals in each age group are: 25-40 (31 carriers, 2094 non-carriers), 41-50 (43 carriers, 2753 non-carriers), 51-60 (38 carriers, 2373 non-carriers) and 61-70 (22 carriers, 1287 non-carriers). Mean ages of subjects in each age range are as given: 25-40 (37.3 ± 2.6), 41-50 (47.5 ± 2.2), 51-60 (56.8 ± 2.6) and 61-70 (66.1 ± 2.8). Males and females are equally represented in each group.

Orthologs and paralogs

Full length orthologous protein sequences from a range of metazoan species (including primates, rodents, birds, fish, *Drosophila melanogaster*, sea urchin (*Strongylocentrotus purpuratus)* and *C. elegans*) were extracted from GenBank. These were confirmed as orthologs based on database annotation of identity and/or predicted function, as well as the requirement that the sequence is the top hit in a BLAST of the human sequence against the genome database for each organism. Conservation at selected positions was further checked and confirmed by BLAST searches of the sequence against the genomic and EST database of that organism. The human NCCT sequence was aligned to the following vertebrate orthologs: mouse, rat, rabbit, dog, cow, chicken, zebrafish and winter flounder. The NKCC2 human sequence was aligned to mouse, rat, rabbit, dog, chicken, zebrafish and *Tetraodon*. These two evolutionarily and functionally related proteins share a single common ancestor in *S. purpuratus, C.elegans* and *D. melanogaster*. Sequences were also compared among paralogs of similar function: NCCT/SLC12A3 and NKCC2/SLC12A1 sequences were aligned to each other and to the close paralog human NKCC1/SLC12A2, as well as to the more distant functional paralogs of the SLC12A family KCC1-4 or SLC12A4-7. ROMK orthologs included were human, mouse, rat, dog, chicken, cow, frog, zebrafish, fugu, *C.elegans*, sea urchin and *D. melanogaster*. Human paralogs analyzed were KCNJ2-6, KCNJ8-16. Multiple and pairwise alignments were done using CLUSTALW. Mutations were inferred as functional if they occur at residues completely conserved in orthologs; the only exception to this is when the mutant residue is found at the homologous position in distant paralogs, since that would imply that the new residue is tolerated at that position and that it is unlikely to cause loss of function.

Evaluation of criteria for functional mutations

We compared the set of 246 Gitelman syndrome (GS) mutations from independent European Caucasian kindreds to the set of variants found in FHS unrelateds with MAF <1%. Using a disease allele frequency of 1/200[16,46], the population frequency of each Gitelman mutation was estimated. We then estimated the sensitivity and specificity of various criteria of evolutionary conservation for identification of loss of function mutations. Sensitivity was estimated based on the percentage of Gitelman alleles captured by each criterion. The corresponding specificity of criteria was estimated via application of Bayes' theorem:

$$P(A|B) = P(B|A) \quad P(A) \; / \; P(B)$$

where the probability that an allele found in a FHS subject is disease causing (A) given that it is of type *n* (B) equals the proportion of alleles of type *n* among Gitelman mutations, divided by the proportion of alleles of type *n* in FHS multiplied by the frequency of Gitelman syndrome alleles in the population.

Statistical analyses

In statistical comparisons of adjusted BP levels between carriers and non-carriers, correlations between relatives (siblings and first-cousins) were taken into account. We used a two-tailed t-test to test the null hypothesis that the mean BP level is 0 for carriers, and adjusted the standard error in the t-test by an inflation factor as given by

$$\sqrt{\frac{n_c+2n_{sp}c_{sibs}+2n_{fcp}c_{fc}}{n_c}},$$

where n_c, n_{sp}, and n_{fcp} represent the number of carriers, the number of sibling pairs who are both carriers, and the number of first cousin pairs who are both carriers, respectively, and c_{sibs} and c_{fc} are the covariances between the siblings and first-cousins in the Framingham population in general.

The inflation factor is calculated as follows. Let X_i denote the trait value of the ith individual in the sample, and $\bar{X}$ the mean of their trait values. Then in the presence of sibling and first-cousin pairs, under the null hypothesis of no association between the mutations and trait values,

$$Var\left(\overline{X}\right)=\left(\Sigma_{i=1}^{n}\mathrm{var}\left(X_i\right)+2\Sigma_{i=1}^{n-1}\Sigma_{j=i+1}^{n}\mathrm{cov}\left(X_i,X_j\right)\right)/n^2=\left(\Sigma_{i=1}^{n}\mathrm{var}\left(X_i\right)+2n_{sp}c_{sibs}+2n_{fcp}c_{fc}\right)/n^2$$

In standard t-test where the relatedness among sampled individuals is not considered, the variance of $\bar{X}$ would be estimated by, $\left(\Sigma_{i=1}^{n}\mathrm{var}\left(X_i\right)\right)/n^2$ so the ratio between the correct variance and $\left(\Sigma_{i=1}^{n}\mathrm{var}\left(X_i\right)\right)/n^2$ is

$$\left(\Sigma_{i=1}^{n}\mathrm{var}\left(X_i\right)+2n_{sp}c_{sibs}+2n_{fcp}c_{fc}\right)/\left[\Sigma_{i=1}^{n}\mathrm{var}\left(X_i\right)\right]$$

Because the traits are normalized, we have

$$\left(n+2n_{sp}c_{sibs}+2n_{fcp}c_{fc}\right)/n$$

The use of a t-test was justified since the distribution does not violate the assumption of a normal distribution using the Kolmogorov-Smirnov test (P = 0.725 and 0.917 for long term average SBP and DBP respectively). An inflation factor of similar form was used for the binomial test where the null hypothesis is that the carrier status does not change the probability of a subject having an adjusted BP reading below or above the population mean.

To further control for family effects, we tested the null hypothesis that the variation in adjusted SBP levels among mutation carriers was no different from the variation among all members in the same family. A statistically significant result would indicate that carriers have more similar adjusted SBP level. A two-tailed paired t-test was used to compare mean SBP between carrier and non-carrier siblings. We considered the ratio of the variance of the carrier sibs versus the variance of all siblings in each family. 6 sibships each having at least 1 carrier-carrier pair and 1 carrier-non carrier pair were considered. The variance ratio was then averaged across families as the test statistic. To derive the empirical distribution of the test statistic under the null hypothesis, 10,000 permutations were performed by randomizing carrier and non-carrier status of siblings within each family and recalculating the averaged

ratio each time. This enabled estimation of the probability of a ratio less than or equal to the observed ratio occurring by chance.

Kaplan-Meier analysis of the time to onset of hypertension was performed on the first age at which hypertension was diagnosed. A log-rank test and Cox proportional hazard analysis was used to compare carriers and non-carriers.

In all analyses, $\alpha = 0.05$ was used as the threshold for significance in two-tailed tests. Statistical analyses were done using R 2.4.1, GraphPad Prism 4 and SPSS 14.0.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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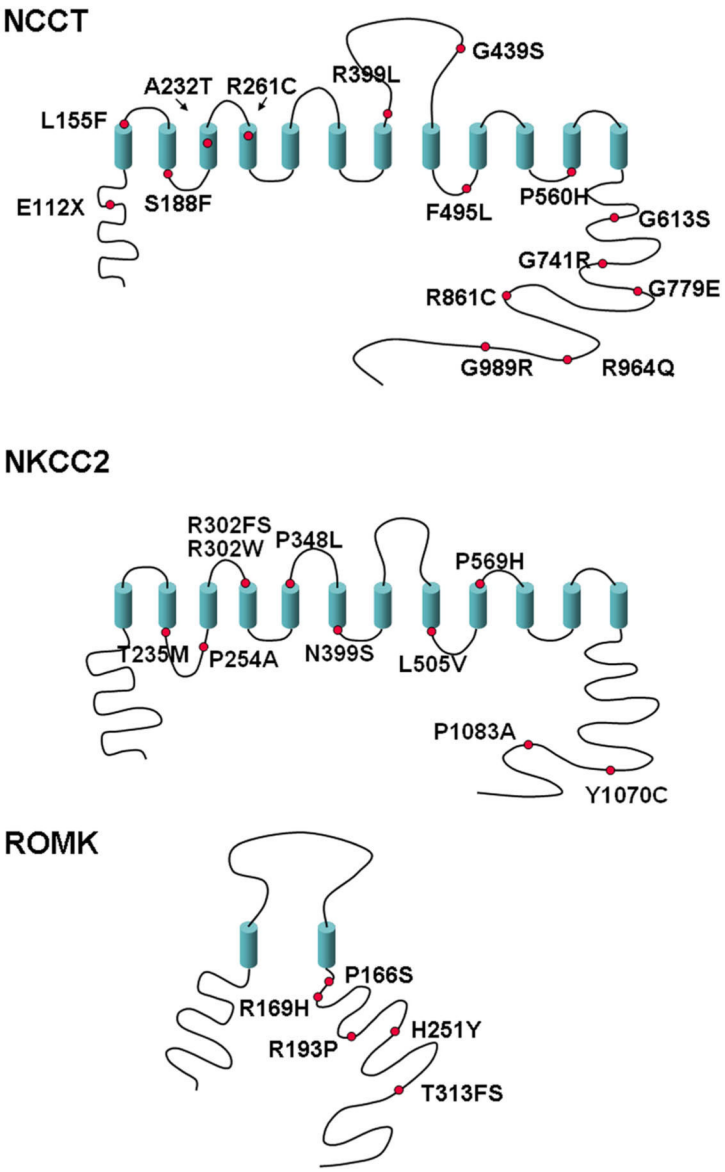

Figure 1. Proven and inferred mutations in *NCCT, NKCC2* and *ROMK* in the Framingham Heart Study offspring cohort

The predicted structure of each protein is shown, with locations of putatively functional missense variants found in FHS indicated in single letter code (X = premature termination; FS = frameshift).

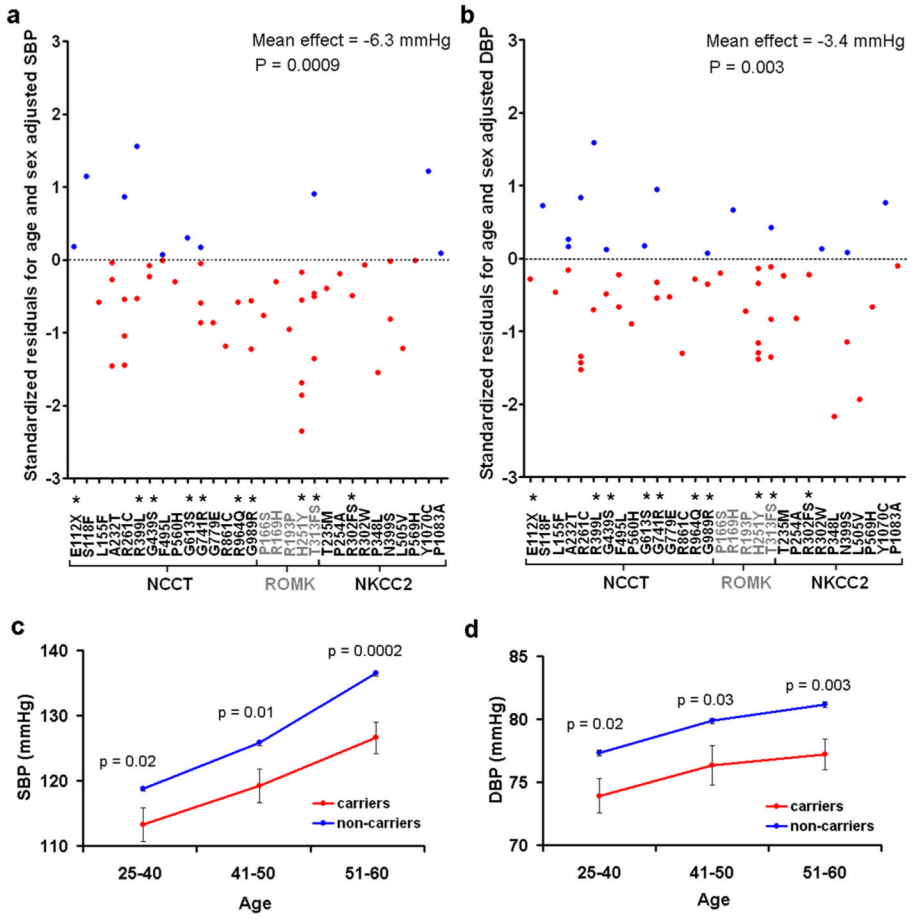

Figure 2. Heterozygous mutations in *NCCT, NKCC2* and *ROMK* lower blood pressure

a) and b) show distributions of standardized residual values for age and sex-adjusted long-term average blood pressure among carriers of putative functional mutations. Adjusted values for the cohort have a mean value of zero and a standard deviation 1. Mean effect size and parametric P-values are indicated. a) Systolic blood pressure. b) Diastolic blood pressure. Panels c) and d) show the mean and standard error of systolic (c) and diastolic (d) blood pressures among mutation carriers (n ranges from 31 to 43 in different decades) and non-carriers (n= 2094-2753) at the last examination in different age groups. P-values comparing values of mutation carriers and non-carriers by t-tests are shown. Asterisks indicate biochemically proven loss of function mutations.

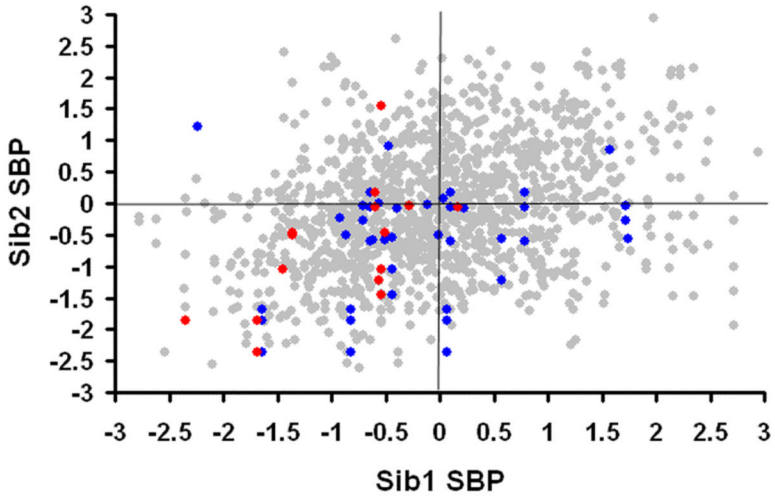

Figure 3. Correlation of blood pressures among sibling pairs in Framingham Heart Study

The distribution of standardized SBP residuals of all 1369 sibling pairs in the FHS cohort is shown. The blood pressures of sibling pairs concordant for putative functional mutations (red), discordant sibling pairs (blue) and those with no mutations (gray) are indicated. For discordant pairs, the sibling carrying the mutation is represented on the y-axis (sib2).

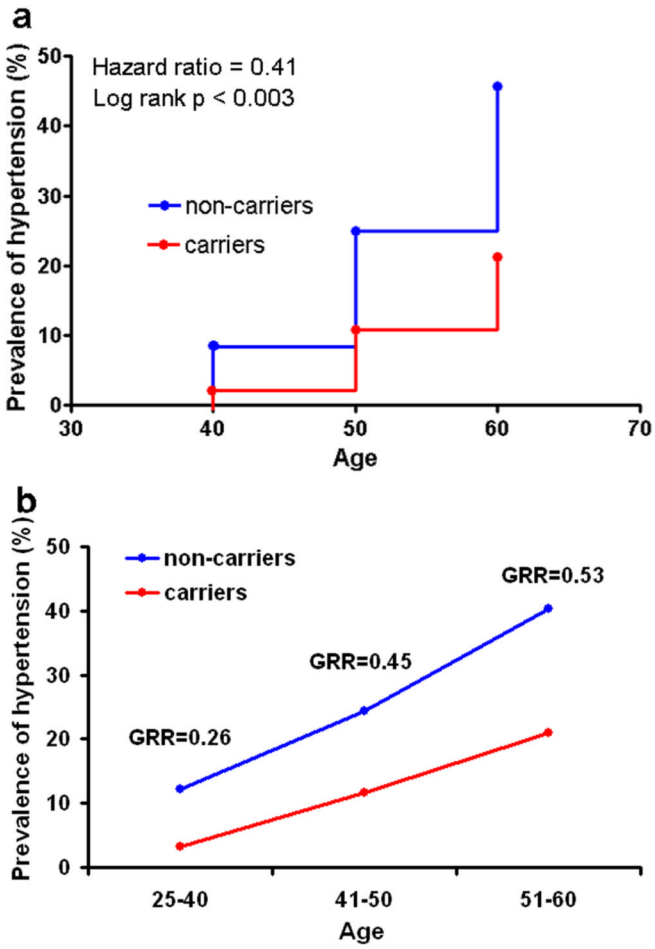

Figure 4. Reduced prevalence of hypertension among mutation carriers

a) Kaplan-Meier plot of time to onset of hypertension in the 49 carriers and 3076 non-carriers. b) The prevalence of hypertension at the last exam within age 25-40, 41-50 and 51-60 years is shown for mutation carriers and non-carriers. The genotype relative risk (GRR) for mutation carriers is shown.

Table 1

Proven and inferred mutations in *NCCT, NKCC2* and *ROMK* in FHS.

Variant	Phylogenetic conservation of wild type residue	Allele frequency in FHS unrelateds	Allele frequency in European Caucasian disease patients	Biochemical loss of function *18-22*	SIFT/Polyphen
NCCT					
E112X*	disruptive	0.00025	0.0039	yes	NA
L155F	complete	0.00025	0	−	++/+
S188F	complete	0.00025	0	−	++/++
A232T	complete	0.00050	0	−	++/+
R261C*	complete	0.00050	0	−	++/++
R399L*	vertebrate	0.00025	0.0078	yes	++/++
G439S*	complete	0.00050	0.0391	yes	++/++
F495L	complete	0.00025	0	−	++/++
P560H*	complete	0.00025	0	−	++/++
G613S*	complete	0.00025	0.0039	yes	++/++
G741R*	complete	0.00050	0.1211	yes	++/++
G779E	complete	0.00025	0	−	++/++
R861C*	vertebrate	0.00025	0.0195	−	++/+
R964Q*	vertebrate	0.00025	0.0117	yes	++/++
G989R*	vertebrate	0.00025	0.0156	yes	−/+
NKCC2					
T235M	complete	0.00025	0	−	++/++
P254A	complete	0.00025	0	−	++/++
R302-FS*	disruptive	0.00025	0.0069	yes	NA
R302W	complete	0.00025	0	−	++/++
P348L	complete	0.00025	0	−	++/++
N399S	vcomplete	0.00050	0	−	++/++
L505V	complete	0.00025	0	−	++/+
P569H	complete	0.00025	0	−	++/++
Y1070C	complete	0.00025	0	−	++/++
P1083A	complete	0.00025	0	−	++/++
ROMK					
P166S	complete	0.00025	0	−	++/+
R169H	complete	0.00025	0	−	++/++
R193P	complete	0.00025	0	−	++/++
H251Y	complete	0.00050	0	yes	++/++
T313-FS*	disruptive	0.00025	0.0104	yes	NA

++: probably damaging (PolyPhen) or deleterious (SIFT); +: possibly damaging or deleterious/low confidence; −: benign or tolerated.

*Previously reported as disease-causing.

Table 2

Effects of mutations on blood pressure phenotypes.

		All mutation carriers (N = 49)	P-value	Carriers with proven mutations (N = 23)	P-value	Carriers with inferred mutations (N = 26)	P-value
% below long-term mean SBP		80	0.001	78	0.05	81	0.009
Mean effect of mutations on:	**Long-term average SBP**	−6.3 mmHg	0.0009	−6.8 mmHg	0.03	−5.9 mmHg	0.01
	SBP at age 60	−9.0 mmHg	0.0002	−9.8 mmHg	0.02	−8.6 mmHg	0.002
	SBP difference between carrier and non-carrier siblings	−6.6 mmHg	0.009	−9.2 mmHg (N = 29 pairs)	0.002	−1.3 mmHg (N = 14 pairs)	0.79
	Mean difference in SBP from FHS mean for carrier-carrier sibling pairs	−11.5 mmHg	0.0001	−11.6 mmHg (N = 11 pairs)	0.001	−11.2 mmHg (N = 4 pairs)	0.06
Kaplan-Meier risk of hypertension in mutation carriers compared with non-carriers		0.41	0.003	0.55	0.13	0.32	0.007

Table 3

Distribution of functional mutation carriers within the population distribution for longitudinal SBP.

	Bottom 10%	Top 10%	Bottom 20%	Top 20%	Bottom 30%	Top 30%	Bottom 40%	Top 40%	*Bottom 50%	Top 50%
NCCT Carriers (%)	3 (11.1)	1 (3.7)	6 (22.2)	3 (11.1)	10 (37.0)	3 (11.1)	13 (48.2)	5 (18.5)	20 (74.1)	7 (25.9)
NKCC2 Carriers (%)	2 (18.2)	0 (0)	2 (18.2)	1 (9.1)	3 (27.3)	1 (9.1)	5 (45.4)	1 (9.1)	9 (81.8)	2 (18.3)
ROMK Carriers (%)	4 (33.3)	0 (0)	5 (41.7)	1 (8.3)	6 (50.0)	1 (8.3)	9 (75.0)	1 (8.3)	11 (91.7)	1 (8.3)
All Carriers (%)	9 (18.4)	1 (2.0)	13 (26.5)	5 (10.2)	19 (38.8)	5 (10.2)	27 (55.1)	7 (10.2)	39 (79.6)	10 (20.4)
Non-Carriers	304	312	613	621	920	934	1225	1245	1633	1443
Fisher's exact/Chi square	P = 0.02		P = 0.09		P = 0.006		P = 0.0008		P = 0.0002	

*Based on numbers of carriers and non-carriers above and below the population mean of 0.